Research Article

Received: 31 December 2007 Revised: 28 July 2008 Accepted: 26 August 2008 Published online in Wiley Interscience: 16 October 2008

(www.interscience.com) DOI 10.1002/jsfa.3418

Cell wall fermentation kinetics are impacted more by lignin content and ferulate cross-linking than by lignin composition

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Abstract

BACKGROUND: We used a biomimetic model system to ascertain how reductions in ferulate–lignin cross-linking and shifts in lignin composition influence ruminal cell wall fermentation. Primary walls from maize cell suspensions with normal or reduced feruloylation were artificially lignified with various monolignols previously identified in normal, mutant, and transgenic plants. Cell wall fermentability was determined from gas production during *in vitro* incubation with rumen microflora and by analysis of non-fermented polysaccharides.

RESULTS: Hemicellulose fermentation lag time increased by 37%, rate decreased by 37%, and the extent declined by 18% as cell wall lignin content increased from 0.5 to 124 mg g^{-1}. Lignification increased lag time for cellulose fermentation by 12-fold. Ferulate–lignin cross-linking accounted for at least one-half of the inhibitory effect of lignin on cell wall fermentation. Incorporating sinapyl *p*-coumarate, a precursor of *p*-coumaroylated grass lignin, increased the extent of hemicellulose fermentation by 5%. Polymerizing varying ratios of coniferyl and sinapyl alcohols or incorporating 5-hydroxyconiferyl alcohol, coniferaldehyde, sinapyl acetate, or dihydroconiferyl alcohol into lignin did not alter the kinetics of cell wall fermentation.

CONCLUSION: The results indicate that selection or engineering of plants for reduced lignification or ferulate–lignin cross-linking will improve fiber fermentability more than current approaches for shifting lignin composition.

Keywords: cell wall; lignin, ferulate; digestibility; fermentability

INTRODUCTION

Lignification of the plant cell wall matrix hinders the enzymatic breakdown of structural polysaccharides, impairing lignocellulosic conversion into fuel ethanol and feed utilization by livestock. Selection or engineering of plants for reduced lignin content often dramatically improves the saccharification and fermentation of structural polysaccharides, but often this is accompanied by dramatic shifts in lignin composition.[1,2] As a result, it is not clear whether or not shifts in lignin composition contribute to observed improvements in fiber degradability.

In addition to shifts in the main monolignol precursors (coniferyl alcohol **1** and sinapyl alcohol **2**, Fig. 1), plants with perturbed monolignol biosynthesis can incorporate high levels of unusual phenylpropanoid precursors (e.g., **3–5**) into lignin.[3] In addition, some plants naturally incorporate high levels of monolignol esters such as sinapyl acetate (**6**).[4,5] Grass lignins are extensively esterified with terminal *p*-coumarate units, which are mainly derived from sinapyl *p*-coumarate (**7**) incorporation into lignin.[6–8] Ferulate (**8**) and a wide array of its coupling products (e.g., **9**), although esterified to arabinoxylans, also become components of grass lignins due to their active copolymerization with monolignols throughout primary and secondary cell wall formation.[9–12] More significantly, homocoupling of ferulates and heterocoupling of ferulates with monolignols or lignin oligomers extensively interconnects the cell wall matrix by cross-linking arabinoxylan chains (**9**) and by cross-linking arabinoxylans to lignin (**10**).

Studies with a biomimetic cell wall model system[13] and mutant or transgenic plants[1,14] indicate guaiacyl lignin units formed from coniferyl alcohol and syringyl lignin units formed from sinapyl alcohol have comparable inhibitory effects on cell wall degradation by cellulolytic enzymes or rumen micro-organisms. Although normally a minor lignin component, hydroxyphenyl units derived from *p*-coumaryl alcohol probably have analogous effects on cell wall degradability.[13] In contrast, other model studies suggest that substituting coniferyl alcohol with high

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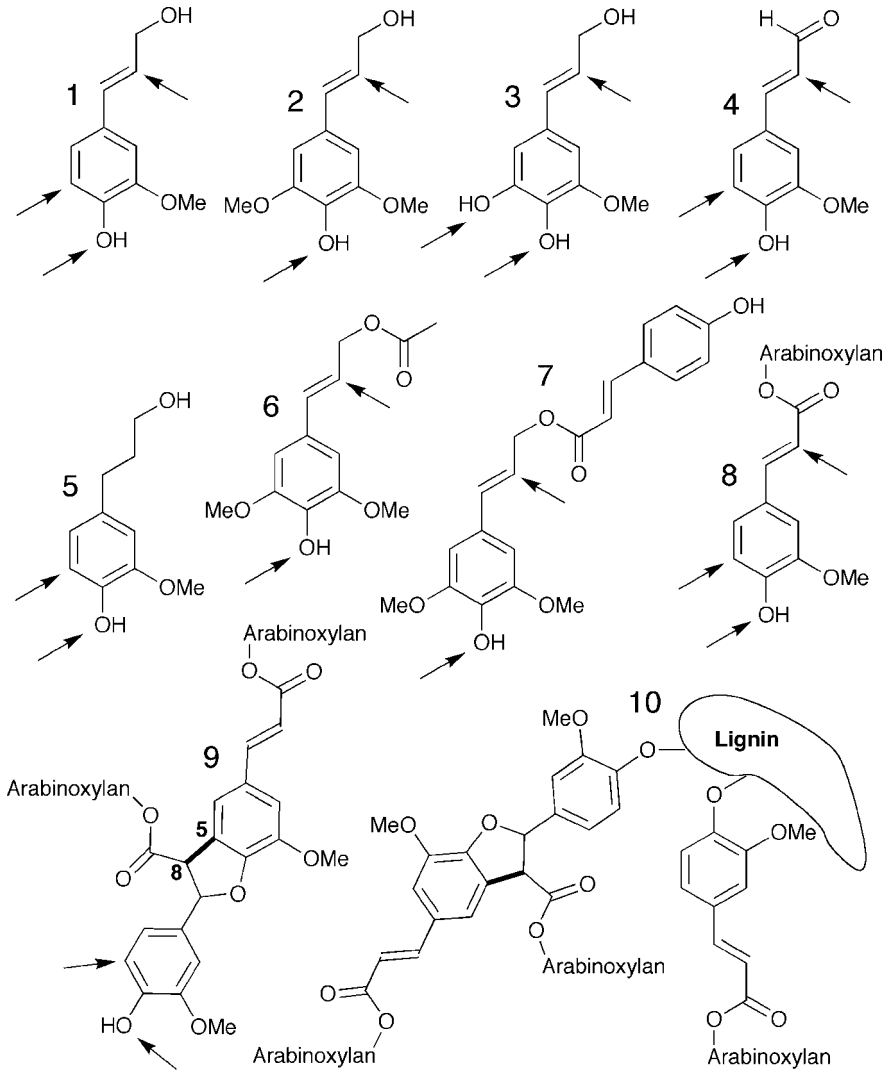

Figure 1. Monolignol precursors (**1–7**) and ferulate structures (**8–10**) examined in studies with maize cell walls. In addition to coniferyl alcohol **1** and sinapyl alcohol **2**, angiosperm lignin can contain elevated levels of 5-hydroxyconiferyl alcohol **3**, coniferaldehyde **4**, dihydroconiferyl alcohol **5**, sinapyl acetate **6**, or sinapyl *p*-coumarate **7**. During lignification, ferulate–arabinoxylan esters **8** undergo oxidative coupling to form diferulate cross-links, e.g., to **9**, and ferulate–lignin cross-links, e.g., to **10**. Arrows indicate potential sites for radical coupling reactions during lignification.

levels of coniferaldehyde **4** in lignin severely depresses the enzymatic and ruminal hydrolysis of cell walls.[15] The effects of incorporating other atypical phenylpropanoid precursors (e.g., **3, 5, 6**) on cell wall degradability are not known. In grasses, reduced cross-linking of arabinoxylans by diferulates and particularly cross-linking of arabinoxylans to lignin by ferulate and diferulates enhanced the degradability of cell walls in model[16,17] and in whole plant studies.[18,19] Co-polymerization of sinapyl *p*-coumarate with monolignols during grass cell wall lignification interferes with ferulate cross-linking of cell walls,[20] but it is not known if this influences cell wall degradability.

In this study, we used the aforementioned cell wall model system to assess how lignification, diverse shifts in lignin composition, and reductions in ferulate–lignin cross-linking influence the *in vitro* kinetics of cell wall fermentation by rumen micro-organisms. This model system provides a biomimetic means of isolating the effects of specific cell wall traits from confounding anatomical, compositional, or structural factors normally encountered in studies with whole plants or isolated plant tissues.[21] Since fermentability of cell walls by rumen micro-organisms is highly correlated with fermentability of cellulosic biomass to ethanol,[22] our results will have broad application in plant selection and engineering efforts aimed at improving the utilization of both ruminant feedstuffs and industrial feedstocks.

MATERIALS AND METHODS

Preparation of cell walls

As described previously,[20] nonlignified cell suspensions of *Zea mays* (L.) were grown for 14 days with 0, 2 or 10 μg mL^{-1} of 2-aminoindan-2-phosphonic acid (AIP) to reduce cell wall feruloylation. Isolated cell walls were stirred in water and treated with H_2O_2 (~2.5 equiv per mol cell wall ferulate) to dimerize ferulates via wall bound peroxidase. Portions of the cell walls were then artificially lignified by gradually adding separate solutions of monolignols (coniferyl alcohol, sinapyl alcohol, and sinapyl *p*-coumarate in molar ratios of 3 : 4 : 0, 3 : 3 : 1 or 3 : 2 : 2) and H_2O_2 (~1.15 equiv per mol monolignols).

In other previously described experiments,[23] cell walls from maize cell suspensions grown without AIP were stirred in Homopipes buffer (25 mmol L^{-1}, pH 5.5) and treated with H_2O_2 (~2.5 equiv per mol cell wall ferulate) to dimerize ferulates via wall bound peroxidase. Separate solutions of monolignols and H_2O_2 (~1.15 equiv per mol monolignols) were then gradually added to artificially lignify cell walls via wall-bound peroxidase. The monolignol mixtures were comprised of varying ratios of coniferyl and sinapyl alcohols or a 1 : 1 : 1 molar ratio of these monolignols with sinapyl acetate, 5-hydroxyconiferyl alcohol, coniferaldehyde or dihydroconiferyl alcohol.

Following precursor additions, cell walls in both experiments were stirred for 24–48 h, collected on glass-fiber filters (1.2 µm porosity), and then washed with water followed by acetone to remove unbound dehydrogenation products. After evaporating off acetone in a hood, cell walls were dried at 55 °C and weighed. To increase the scope of inference of the studies,[24] treatments were replicated by adding two or three levels of precursors to cell walls.

Cell wall and statistical analyses

Acid-insoluble Klason lignin in duplicate cell wall samples was determined by a two-stage hydrolysis in 12 mol L^{-1} H_2SO_4 at 25 °C for 2 h followed by 1.6 mol L^{-1} H_2SO_4 at 100 °C for 3 h.[25] Ester-linked *p*-hydroxycinnamate monomers and dimers released from duplicate cell wall samples by 2 mol L^{-1} NaOH (20 h, 25 °C) were extracted with ethyl ether, silylated and quantified by gas–liquid chromatography with flame ionization detection (GLC-FID).[10,26] Whole cell walls (~40 mg) lignified at the highest precursor level were sonicated in 1–2 mL of DMSO-d_6 and subjected to gel-state nuclear magnetic resonance (NMR) using a cryoprobe 750 MHz (DMX-750) Bruker Biospin (Rheinstetten, Germany) instrument as described by Kim *et al.*[27]

Gas production during fermentation of 100 mg of cell walls at 39 °C in 60 mL sealed bottles was monitored with pressure transducers following addition of 5.7 mL of phosphate–bicarbonate buffer, 0.3 mL of reducing agent, and 4 mL of diluted rumen inoculum.[28] Filtered inoculum was prepared with a 1 : 2 ratio (vol/vol) of rumen fluid and blended buffer-extracted rumen solids collected from two Holstein cows fed a total mixed ration of corn silage, corn grain, alfalfa hay, soybean meal, and supplemental vitamins and minerals.[28] Fermentations were carried out for 45 h in two to four independent runs. Due to time-staggered inoculation of bottles and differences in time intervals between pressure readings, data were not averaged across runs prior to fitting by nonlinear regression. Blank-corrected gas production data were fitted with a dual-pool logistic model to estimate the kinetics of cell wall fermentation.[28] Nonfermented polysaccharides (NFPs) in freeze-dried fermented residues were dissolved in 12 mol L^{-1} H_2SO_4 at 25 °C for 2 h and analyzed by the phenol–sulfuric acid assay[29] with corrections for inoculum contamination and sugar recovery. The recovery of sugars from NFPs was estimated by running unfermented nonlignified cell walls through the 12 mol L^{-1} H_2SO_4 dissolution/phenol–sulfuric acid assay procedure.

Kinetic data were subjected to analysis of variance and, in most cases, Klason lignin content was used as a covariate to adjust means and increase precision.[24] Unless noted otherwise, all reported differences were significant at $P = 0.05$.

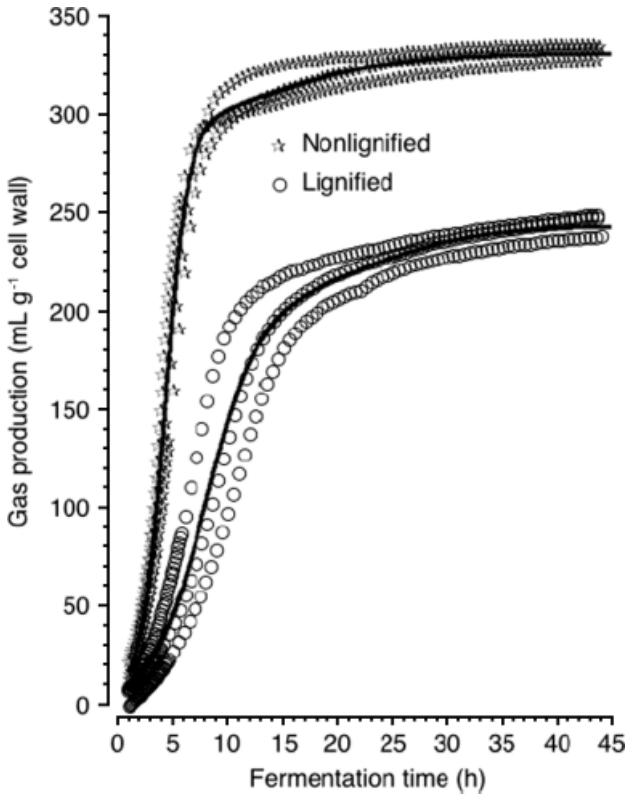

Figure 2. Blank-corrected *in vitro* gas production from nonlignified and lignified cell walls fermented in three independent runs with rumen inoculum. Two-pool logistic curves overlay the data.

RESULTS AND DISCUSSION

Lignification and cell wall fermentability

As described in previous studies,[20,23] a diverse array of artificially lignified cell walls were prepared by gradually adding various monolignols and H_2O_2 to ruptured and buffer-washed primary cell walls of maize. During *in vitro* incubation with mixed rumen microflora, both nonlignified and lignified cell walls showed a biphasic profile of gas production, which was best described with a two-pool logistic model with two discrete lag times (Fig. 2). The goodness of fit (≥0.98) of this logistic model consistently exceeded that of other dual or single pool exponential and logistic models (data not shown). For nonlignified cell walls containing only ~5 mg g^{-1} of Klason lignin,[30] both pools began fermentation ≤2 h after inoculation but the rate and extent of gas production from the primary *A* pool were about six-fold greater than the secondary *B* pool (Table 1). Based on previous fermentation studies of grass cell walls and compositional analyses of nonlignified maize walls,[31–35] the larger *A* pool was probably derived from rapidly fermented hemicellulose (and small amounts of pectin), while the smaller *B* pool would likely be indicative of slowly fermented cellulose. Nonlignified cell walls were extensively degraded and contained only 21 mg g^{-1} of NFPs.

Artificial lignification of cell walls to a Klason lignin content of 124 mg g^{-1} increased lag time by 37%, decreased fermentation rate by 37%, and decreased gas production by 18% from the hemicellulosic *A* pool (Table 1). Lignification dramatically increased lag time for the cellulosic *B* pool from 0.6 to 7.9 h without affecting rate or pool size. Thus lignin mainly depressed the rate and extent of hemicellulose fermentation in cell walls, which is plausible given their intimate association in the cell wall. Previous studies with

Table 1. *In vitro* fermentation kinetics and nonfermented polysaccharide (NFP) content of nonlignified and artificially lignified cell walls of maize

Cell wall type	L_1 (h)*	k_1 (h^{-1})*	A (mL g^{-1})*	L_2 (h)†	k_2 (h^{-1})†	B (mL g^{-1})†	$A+B$ (mL g^{-1})	NFP (mg g^{-1})
Nonlignified	1.98	0.239	284	0.64	0.037	52	336	20.6
Lignified	2.72	0.151	232	7.86	0.040	51	283	83.7
P-value ($n = 6$)	<0.01	<0.01	<0.01	<0.01	0.22	0.73	<0.01	<0.01

Cell walls were lignified with a 3 : 4 molar ratio of coniferyl and sinapyl alcohol to a Klason lignin content of 124 mg g^{-1}. Data were averaged over cell wall treatments containing 2.0–12.3 mg g^{-1} of total ferulates (monomers plus dimers).
* Lag time (L_1), rate constant (k_1), and gas produced (A) for the rapidly digested pool.
† Lag time (L_2), rate constant (k_2), and gas produced (B) for the slowly digested pool.

Table 2. *In vitro* fermentation kinetics and nonfermented polysaccharide (NFP) content of nonlignified cell walls of maize treated with H_2O_2 to increase diferulate cross-linking

Treatment (diferulate level)	L_1 (h)*	k_1 (h^{-1})*	A (mL g^{-1})*	L_2 (h)†	k_2 (h^{-1})†	B (mL g^{-1})†	$A+B$ (mL g^{-1})	NFP (mg g^{-1})
None (1.6 mg g^{-1})	1.98	0.239	284	0.64	0.037	52	336	20.6
H_2O_2 (2.6 mg g^{-1})	2.11	0.229	278	1.12	0.039	49	327	20.8
P-value ($n = 6$)	0.25	0.02	0.07	0.60	0.37	0.37	0.01	0.86

Data were averaged over cell wall treatments containing 2.0–12.3 mg g^{-1} of total ferulates (monomers plus dimers).
* Lag time (L_1), rate constant (k_1), and gas produced (A) for the rapidly digested pool.
† Lag time (L_2), rate constant (k_2), and gas produced (B) for the slowly digested pool.

grass stems and tissue isolates also indicated that lignification reduces the rate and extent of cell wall fermentation, but impacts on hemicellulose fermentation were not consistently greater than cellulose.[34,36] These studies also noted an increase in lag time due to lignification, with the effects being much greater for cellulose than for hemicellulose.

Total gas production declined 16% and NFP increased from 21 to 84 mg g^{-1} when Klason lignin comprised 124 mg g^{-1} of cell walls (Table 1), indicating that simple dilution of structural polysaccharides due to monolignol polymerization into cell walls accounted for most of the decline in fermentability. Similar relationships between cell wall fermentability and Klason lignin may be surmised from other work with grasses.[37,38] Other studies based on acid-detergent lignin assays indicate that lignin protects several times its mass of structural polysaccharides from fermentation.[39] Due to the inclusion of covalently bound ferulates, proteins and polysaccharides, Klason residues tend to overestimate the lignin content of primary maize cell walls.[25,40] Conversely, the acid-detergent procedure severely underestimates lignin in grasses due to loss of lignin–polysaccharide complexes during detergent extraction.[41] More accurate mass balance estimates indicate the actual lignin content of these cell walls was 78 mg g^{-1}.[20] Using this lignin estimate, the aforementioned reduction in gas production and increase in NFPs both indicate there was about a 1 : 1 ratio of lignin to NFPs in lignified walls.

Ferulate cross-linking and cell wall fermentability

Growing maize cell suspensions with AIP to reduce total ferulate (monomer plus dimers) concentrations from 12.3 to 2.0 mg g^{-1} had no impact on fermentation kinetics or NFP levels in nonlignified cell walls (data not shown). In contrast, H_2O_2 treatment to stimulate peroxidase-mediated diferulate formation in nonlignified cell walls depressed the fermentation rate by 4% and gas production by 2% ($P = 0.07$) from the hemicellulosic A pool without affecting lag time or the fermentation kinetics of the cellulosic B pool (Table 2). Greater diferulate cross-linking also reduced total gas production by 3% but no differences in NFP were detected. These results confirm earlier work demonstrating that diferulate cross-linking, not ferulate substitution of xylans, modestly affects the rate, and to a lesser degree, the extent of cell wall enzymatic hydrolysis.[17]

As observed in previous model studies,[16,20] growing maize suspensions with AIP to reduce arabinoxylan feruloylation did not affect the subsequent polymerization of coniferyl and sinapyl alcohols into wall-bound lignin. In cell walls containing 122 mg g^{-1} of Klason lignin, an AIP instigated decrease in ferulate–lignin cross-linking of 85% reduced lag time by 24% and increased fermentation rate by 35% for the hemicellulosic A pool (Table 3). Similar effects on lag time ($P = 0.13$) but not fermentation rate were suggested for the cellulosic B pool. This reduction in cross-linking yielded a modest increase of 6% in total gas production and a corresponding decrease in NFP. These results confirm earlier model work showing that reductions in ferulate–lignin cross-linking enhanced the extent and particularly the rate of cell wall enzymatic hydrolysis.[16] A negative association between ferulate–lignin cross-linking and cell wall degradability has also been observed in grasses.[18,19] In the current study, reductions in ferulate lignin cross-linking yielded linear declines in lag time and quadratic increases in the rate and extent of cell wall fermentation. When extrapolated to cell walls with zero ferulate, these data indicate that ferulate cross-linking may account for roughly one-half of the inhibitory effects of lignin on the lag, rate and extent of cell wall digestion.

Lignin composition and cell wall fermentability

As reported earlier,[20] adding moderate levels of sinapyl *p*-coumarate with monolignols increased Klason lignin concentrations from 100 to 113 mg g^{-1}. At higher precursor levels, sinapyl *p*-coumarate accelerated the inactivation of cell-wall

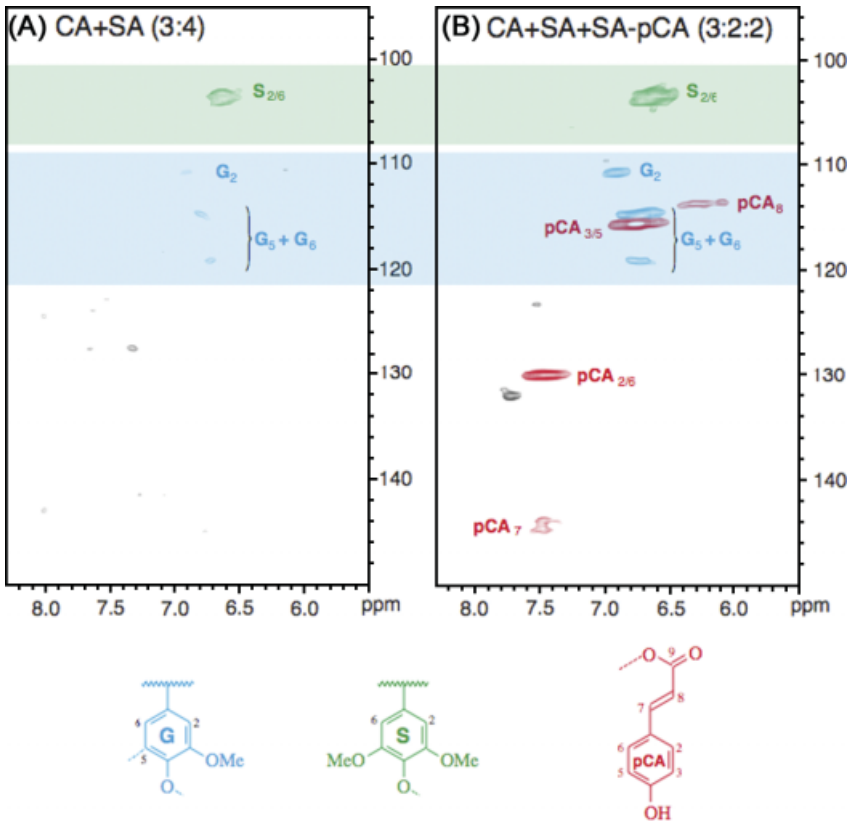

Figure 3. Aromatic regions from $^{13}C-^{1}H$ correlation gel-state 2-D NMR spectra (HSQC) of maize cell walls artificially lignified with (A) coniferyl alcohol (CA) and sinapyl alcohol (SA) in a 3 : 4 molar ratio or (B) CA, SA, and sinapyl *p*-coumarate (SA–pCA) in a 3 : 2 : 2 molar ratio. Correlations from guaiacyl (G), syringyl (S), and pCA units were assigned based on a previous paper.[27]

Table 3. *In vitro* fermentation kinetics and nonfermentable polysaccharide (NFP) content of artificially lignified cell walls containing varying levels of ferulate and diferulate arabinoxylan esters cross-linked to lignin

Ferulate and diferulate cross-linking to lignin	L_1 (h)*	k_1 (h^{-1})*	A (mL g^{-1})*	L_2 (h)†	k_2 (h^{-1})†	B (mL g^{-1})†	$A+B$ (mL g^{-1})	NFP (mg g^{-1})
High (10.9 mg g^{-1})	3.11[a]	0.120[b]	235	10.29	0.042	46	280[b]	92.3[a]
Medium (5.9 mg g^{-1})	2.78[a]	0.131[b]	236	10.48	0.043	46	283[ab]	85.7[a]
Low (1.6 mg g^{-1})	2.35[b]	0.162[a]	247	7.02	0.040	50	297[a]	59.7[b]
P-value ($n = 3$)	0.01	0.02	0.41	0.13	0.68	0.75	0.06	<0.01

Least square means within columns (adjusted to 122 mg g^{-1} Klason lignin by covariance analysis) with unlike letters differ ($P < 0.05$). Data were averaged over treatments lignified with coniferyl alcohol, sinapyl alcohol, and sinapyl *p*-coumarate in molar ratios of 3 : 4 : 0, 3 : 3 : 1 or 3 : 2 : 2.
* Lag time (L_1), rate constant (k_1), and gas produced (A) for the rapidly digested pool.
† Lag time (L_2), rate constant (k_2), and gas produced (B) for the slowly digested pool.

peroxidase and this slightly depressed Klason lignin concentrations from 147 to 131 mg g^{-1}. Concentrations of alkali-labile *p*-coumarate increased from 0.2 to 11.0 mg g^{-1} of cell wall with sinapyl *p*-coumarate addition, confirming that the ester conjugate was extensively co-polymerized with monolignols to form *p*-coumaroylated lignins in cell walls. Further verification of *p*-coumaroylated lignin formation was provided by gel-state two-dimensional (2-D) NMR analysis of whole cell walls (Fig. 3). While absent in cell walls lignified with a 3 : 4 molar ratio of coniferyl and sinapyl alcohols, prominent contours associated with terminal *p*-coumarate units were observed when sinapyl *p*-coumarate replaced one-half of the sinapyl alcohol in the monolignol mixture.

At an adjusted Klason lignin content of 122 mg g^{-1}, co-polymerization of sinapyl *p*-coumarate with monolignols modestly increased gas production by 5% from the hemicellulosic *A* pool without significantly affecting other kinetic parameters or the quantity of NFPs (Table 4). Although sinapyl *p*-coumarate reduced ferulate incorporation into lignin by 10%,[20] this modest reduction in cross-linking would not account for the increased fermentability of the hemicellulosic *A* pool and the lack of effect on other kinetic parameters. Why *p*-coumarate esters render lignin less inhibitory to hemicellulose fermentation is not known and requires further study and confirmation.

Altering the makeup of monolignols added to cell walls had a considerable impact on the quantity and composition of lignin

Table 4. *In vitro* fermentation kinetics and nonfermentable polysaccharide (NFP) content of maize cell walls artificially lignified with coniferyl alcohol, sinapyl alcohol, and varying proportions of sinapyl *p*-coumarate

Proportion of precursors as sinapyl *p*-coumarate	L_1 (h)[a]	k_1 (h^{-1})*	A (mL g^{-1})*	L_2 (h)†	k_2 (h^{-1})†	B (mL g^{-1})†	$A+B$ (mL g^{-1})	NFP (mg g^{-1})
None	2.74	0.151	234[b]	7.95	0.040	50	283	82.6
Low (0.14 mol mol^{-1})	2.83	0.131	238[b]	9.50	0.043	48	287	76.3
High (0.28 mol mol^{-1})	2.67	0.130	246[a]	10.33	0.042	44	290	78.6
P-value ($n = 3$)	0.83	0.19	0.01	0.42	0.71	0.56	0.63	0.61

Least square means within columns (adjusted to 122 mg g^{-1} Klason lignin by covariance analysis) with unlike letters differ ($P < 0.05$). Data were averaged over treatments with high, medium, and low levels of ferulate and diferulate cross-linking to lignin.
* Lag time (L_1), rate constant (k_1), and gas produced (A) for the rapidly digested pool.
† Lag time (L_2), rate constant (k_2), and gas produced (B) for the slowly digested pool.

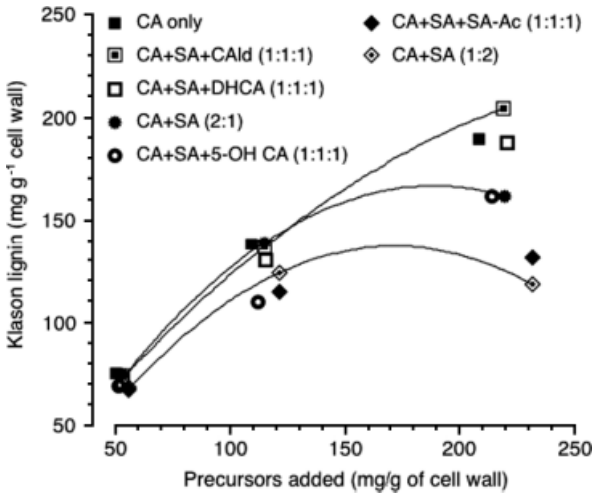

Figure 4. Klason lignin content of maize cell walls artificially lignified with various molar ratios of coniferyl alcohol (CA), sinapyl alcohol (SA), sinapyl acetate (SA–Ac), 5-hydroxyconiferyl alcohol (5-OH CA), coniferaldehyde (CAld), or dihydroconiferyl alcohol (DHCA).

formed (Figs 4 and 5). Shifting from 100% coniferyl alcohol to a 1 : 2 ratio of coniferyl to sinapyl alcohol (or sinapyl acetate) progressively depressed Klason lignin concentrations, but considerably increased syringyl lignin correlation contours detected by gel-state 2-D NMR. When added in 1 : 1 : 1 molar ratio with coniferyl and sinapyl alcohols, coniferaldehyde or dihydroconiferyl alcohol yielded high concentrations of Klason lignin, while intermediate results were obtained with 5-hydroxyconiferyl alcohol. Following lignification, gel-state 2-D NMR readily detected correlation contours resulting from coniferaldehyde, dihydroconiferyl alcohol, and 5-hydroxyconiferyl alcohol in cell walls. When adjusted by covariance analysis to a similar Klason content of 119 mg kg^{-1}, cell walls lignified with the various monolignol mixtures had similar quantities of ferulate monomers plus dimers cross-linked to lignin (14 mg g^{-1}) and similar fermentation characteristics (Table 5). Thus while monolignol composition may influence lignin formation in cell walls, the compositional shifts we examined had no direct impact on cell wall fermentation by rumen microflora. In earlier model studies,[13] lignin formed with varying proportions of normal monolignols (*p*-coumaryl, coniferyl and sinapyl alcohols) similarly had no effect on the enzymatic hydrolysis of cell walls. While not observed in the current study, lignins formed with higher proportions (≥50%) of coniferaldehyde were highly hydrophobic and inhibitory to enzymatic or ruminal degradation of cell walls.[15] Other studies with transgenic plants also indicate that perturbations in the lignin pathway primarily affect the enzymatic degradability of cell walls through shifts in lignin content while shifts in lignin composition may in some cases play a secondary role.[1] In this case, shifts in lignin composition may simply

Table 5. *In vitro* fermentation kinetics and nonfermentable polysaccharide (NFP) content of maize cell walls artificially lignified with varying ratios of coniferyl alcohol (CA), sinapyl alcohol (SA), sinapyl acetate (SA–Ac), 5-hydroxyconiferyl alcohol (5-OH CA), coniferaldehyde (CAld) or dihydroconiferyl alcohol (DHCA)

Monolignols (molar ratio)	L_1 (h)*	k_1 (h^{-1})*	A (mL g^{-1})*	L_2 (h)†	k_2 (h^{-1})†	B (mL g^{-1})†	$A+B$ (mL g^{-1})	NFP (mg g^{-1})
CA only	2.92	0.105	233	11.0	0.096	41	274	101.4
CA + SA (2 : 1)	3.08	0.100	250	16.4	0.056	35	285	83.8
CA + SA (1 : 2)	3.00	0.091	239	16.1	0.056	35	274	95.7
CA + SA + SA–Ac (1 : 1 : 1)	3.00	0.100	248	15.9	0.057	30	278	92.8
CA + SA + 5-OH CA (1 : 1 : 1)	2.61	0.117	244	14.6	0.067	33	277	73.1
CA : SA : CAld (1 : 1 : 1)	3.23	0.109	236	16.5	0.039	43	279	110.8
CA + SA + DHCA (1 : 1 : 1)	3.65	0.116	227	14.7	0.043	40	267	100.6
P-value ($n = 3$)	0.18	0.21	0.16	0.34	0.42	0.71	0.11	0.07

Least square means ($n = 3$) within columns were adjusted to 122 mg g^{-1} Klason lignin by covariance analysis.
* Lag time (L_1), rate constant (k_1), and gas produced (A) for the rapidly digested pool.
† Lag time (L_2), rate constant (k_2), and gas produced (B) for the slowly digested pool.

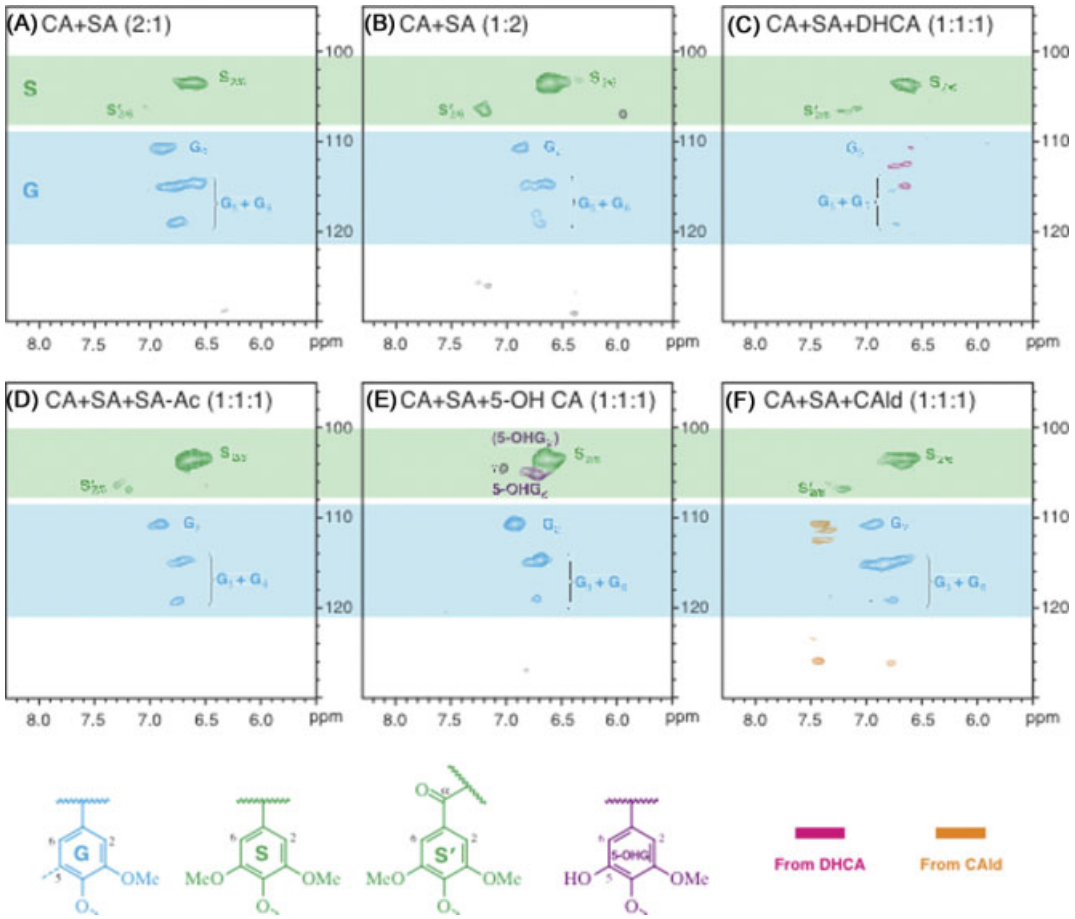

Figure 5. Aromatic regions from $^{13}C-^{1}H$ correlation gel-state 2-D NMR spectra (HSQC) of whole cell walls in DMSO-d_6. Maize cell walls were artificially lignified with various molar ratios of coniferyl alcohol (CA), sinapyl alcohol (SA), dihydroconiferyl alcohol (DHCA), sinapyl acetate (SA–Ac), 5-hydroxyconiferyl alcohol (5-OH CA), and coniferaldehyde (CAld). Correlations associated with guaiacyl (G), syringyl (S or S′), 5-OHG, DHCA, and CAld units were assigned based on a previous paper[27] and using an NMR database.[42].

be associated with changes in other factors (i.e., anatomical or other cell wall traits), which in fact influence cell wall degradability. Lignin composition may, however, play a greater role in controlling cell wall degradation in environments (e.g., the human colon) with less potent polysaccharidase activity.[43]

CONCLUSIONS

In the absence of anatomical constraints or other confounding factors, lignification of cell walls delayed the fermentation of hemicellulose and especially cellulose. Lignification also depressed the rate and extent of hemicellulose fermentation. Conversely, reductions in ferulate cross-linking of arabinoxylans to lignin enhanced the fermentation of hemicellulose. Based on our data, we tentatively estimate that ferulate cross-linking accounted for nearly one-half of the inhibitory effects of lignin on cell wall fermentation. For unknown reasons, sinapyl *p*-coumarate incorporation slightly enhanced the extent of hemicellulose fermentation while other changes in monolignol composition had no effect on fermentation kinetics. Overall, our results indicate that continued breeding or engineering of plants for reduced lignification or ferulate cross-linking will improve fiber fermentability more than perturbing the biosynthesis of known monolignols solely for the purpose of altering lignin composition. While needing further validation, expression of sinapyl *p*-coumarate in forage legumes (which do not innately form *p*-coumaroylated lignins) may provide an alternate route for further improving cell wall fermentability where substantial reductions in lignin content jeopardize plant viability.

ACKNOWLEDGEMENTS

The authors thank Christy Davidson and Len Strozinski for skill and persistence in running cell wall fermentations and Ronald Hatfield and Jane Marita for their assistance with ferulate and *p*-coumarate analysis. This work was supported in part by USDA-NRI (1994-37500-0580, 1996-35304-3864, and 2003-35103-13834) and H. Wilhelm Schaumann Stiftung. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

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